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**Title:** Effect of exercise training on skeletal muscle cytokine expression in the elderly.

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## Abstract

Aging is associated with increased circulating pro-inflammatory and lower anti-inflammatory cytokines. Exercise training, in addition to improving muscle function, reduces these circulating pro-inflammatory cytokines. Yet, few studies have evaluated changes in the expression of cytokine within skeletal muscle after exercise training. The aim of the current study was to examine the expression of cytokines both at rest and following a bout of isokinetic exercise performed before and after 12 weeks of resistance exercise training in young ( $n=8$ ,  $20.3 \pm 0.8$  yrs) and elderly men ( $n=8$ ,  $66.9 \pm 1.6$  yrs). Protein expression of various cytokines was determined in muscle homogenates. The expression of MCP-1, IL-8 and IL-6 (which are traditionally classified as 'pro-inflammatory') increased substantially after acute exercise. By contrast, the expression of the anti-inflammatory cytokines IL-4, IL-10 and IL-13 increased only slightly (or not at all) after acute exercise. These responses were not significantly different between young and elderly men, either before or after 12 weeks of exercise training. However, compared with the young men, the expression of pro-inflammatory cytokines 2 h post intense exercise tended to be greater in the elderly men prior to training. Training attenuated this difference. These data suggest that the inflammatory response to unaccustomed exercise increases with age. Furthermore, regular exercise training may help to normalize this inflammatory response, which could have important implications for muscle regeneration and adaptation in the elderly.

**Key words:** sarcopenia, aging, myokine, chemokine, inflammation, strength, hypertrophy

## Introduction

From approximately the fifth decade of life, there is a gradual and inevitable decline in muscle mass (known as 'sarcopenia') in susceptible individuals (Hughes et al., 2001; Janssen and Ross, 2005). This loss of muscle mass is a major contributor to detrimental health outcomes, including increased falls, frailty and mortality risk (Janssen, 2006; Rantanen et al., 2003). The mechanisms underpinning this loss of muscle are complex, but may include age-related changes in immune function (previously described as inflamm-aging (Franceschi et al., 2000). Inflamm-aging was initially described as an increase in circulating concentrations of classically pro-inflammatory cytokines. However, there are many complex alterations in the adaptive and innate immunity that also influence the secretion of anti-inflammatory and pro-resolving cytokines (Franceschi et al., 2007).

In the elderly, elevated inflammatory cytokines, including TNF- $\alpha$  and IL-6, correlate with reduced muscle mass and strength (Pedersen et al., 2003; Visser et al., 2002). Both *in vitro* and rodent models demonstrate a capacity for these predominantly pro-inflammatory cytokines to increase skeletal muscle protein degradation (Frost et al., 2003; Haddad et al., 2005). In addition, protein degradation in response to treatment with pro-inflammatory cytokines is greater in muscle cells isolated from older individuals compared with muscle cells from younger individuals (Lees et al., 2009; Merritt et al., 2013). It is increasingly recognized that skeletal muscle itself is an important source of inflammatory mediators, collectively known as 'myokines'. Expression of these locally generated cytokines (e.g., IL-1 $\beta$ , TNF- $\alpha$ , IL-1ra, IL-10) is elevated in skeletal muscle of elderly individuals, perhaps as part of

the inflamm-aging process (Buford et al., 2010; Leger et al., 2008; Przybyla et al., 2006; Thalacker-Mercer et al., 2010).

The concept of the anti-inflammatory effects of exercise training has gained increasing attention in recent years (Gleeson et al., 2011). The cytokine species exerting predominantly anti-inflammatory effects (e.g., IL-4, IL-10 and IL-13) (Prokopchuk et al., 2007) also enhance myogenesis (Deng et al., 2012; Heredia et al., 2013). Thus, it is possible that by enhancing the secretion of anti-inflammatory cytokines in skeletal muscle, exercise may promote muscle hypertrophy—or at least limit muscle atrophy—in the elderly.

To date, most studies examining the effects of exercise training on the dynamic regulation of cytokines have focused either primarily on circulating cytokine concentrations, or have analyzed cytokine gene expression within skeletal muscle at rest before and after a period of training (Gielen et al., 2003; Greiwe et al., 2001; Lambert et al., 2008; Nader et al., 2010). The aim of the present study was to compare the protein expression of cytokines in skeletal muscle between young and elderly men in response to a single bout of isokinetic exercise performed before and after 12 weeks of regular resistance training. We hypothesized that (1) the cytokine response to isokinetic exercise before training would be smaller in elderly men compared with young men, and that (2) the cytokine response to isokinetic exercise after training would be similar in elderly and young men.

## **Materials and methods**

### ***Ethics statement***

Prior to written consenting to participate in the study, the nature, purpose and risks of the study were fully explained to all subjects. All experimental procedures involved in this study were formally approved by the Human Research Ethics Committee of Deakin University.

### ***Subjects***

Eight healthy young men (aged 18–25 yrs) and eight healthy elderly men (aged 60–75 yrs) (Table 1), who had not participated in resistance exercise for a minimum of 1 year prior to commencing the study, were recruited. The elderly men completed a comprehensive medical screening procedure, which included a 12-lead ECG exercise stress test to detect any underlying heart conditions prior to the inclusion into the trial.

### ***Experimental Design***

#### ***Single bout of isokinetic exercise***

All subjects completed a familiarization session on the Cybex NORM dynamometer (Cybex International Inc., UK) to become familiar with how to perform the isokinetic exercise. This session involved assessing isokinetic maximal voluntary contraction (MVC) strength and peak torque (Nm) during knee extension/flexion over 12 maximal repetitions at a constant speed of  $60^{\circ}\cdot\text{sec}^{-1}$  in both concentric and eccentric phases. Exercise was performed using the dominant limb. Subjects were instructed to push maximally, and were verbally encouraged throughout the test. Familiarization was performed at least 7 days prior to the first bout experimental trial. For this experimental trial, the subjects arrived at the

laboratory in the fasted state; following 30 min of supine resting, a resting muscle sample was collected. Following the resting biopsy, the subjects completed three sets of 12 repetitions of maximal unilateral knee extension exercise on the dynamometer, with 2 min rest between each set, as described for the familiarization session. Two hours after the completion of the exercise session, another muscle sample was collected. This time point was chosen because inflammatory proteins are most abundant in skeletal muscle at 2 h post exercise (Della Gatta et al, unpublished observations).

### *12 week exercise training*

Following the first experimental trial, subjects completed 12 weeks of fully supervised progressive resistance exercise training on three days each week, with a minimum of 48 h of rest between exercise sessions. Initially, three training sessions were conducted using light resistance for equipment familiarization and correct execution of the exercises. After the familiarization sessions, strength testing was performed to determine appropriate starting weights for all subjects. One repetition maximum (1-RM) strength was estimated using a 5-RM test for all exercises. A 5-RM test was adopted because this exercise test was the most appropriate for an elderly population with no previous history of strength training. At week 6 and week 12, the subjects' 5-RM was retested, and the training load was adjusted accordingly to ensure that the training was progressive.

Each training session was preceded by a 5 min warm-up on a stationary cycle followed by a full set of exercises with light weights. The exercises consisted of leg press, bench press, seated row, leg extension, dumb-bell shoulder press, and sit-ups. Following the warm-up weights, subjects completed two sets at the required intensity, completing between 8 and

12 repetitions on each exercise. Specified rest periods were allowed between sets. Initially, the exercises were set to 50% of individual estimated 1-RM for 1 week, followed by a progressive increase in the weights lifted each week until the subjects were lifting 80% of estimated 1-RM at week 6. The exercise intensity was set at 80% of estimated 1-RM for the remaining 6 weeks.

At the end of the 12 week training program, subjects again visited the laboratory in a fasted state to complete the exercise trial consisting of a single bout of isokinetic exercise and collection of muscle biopsies. The exercise performed and timing of the muscle biopsies were identical to the exercise trial that the subjects completed before their progressive resistance training.

#### ***Muscle Biopsy procedure***

The subjects were required to rest in a supine position for 30 minutes prior to muscle sampling. A muscle sample was then collected from the *vastus lateralis* under local anesthesia (Xylocaine 1%) using the percutaneous needle biopsy technique (Bergström, 1962), including suction (Evans et al., 1982). Excised muscle tissue was immediately frozen and stored in liquid nitrogen for subsequent analysis. To minimize the potential for interference, serial muscle biopsies were collected at least 2 cm from the previous muscle biopsy site. Biopsies were taken at rest and 2 h following the completion of the acute exercise session, before and after 12 weeks of resistance exercise training.

#### ***Multiplex analysis***



A bio-plex assay (Bio-Rad Laboratories, Hercules, CA) was used to analyze the protein expression of cytokines within skeletal muscle tissue. In the present study, kits were designed for the simultaneous analysis of IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1 and TNF- $\alpha$ . The assay was conducted following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Tissue samples (10 mg) were homogenized in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Igepal CA-630, 10  $\mu$ g/ml Aprotinin, 10  $\mu$ g/ml Leupeptin, 3 mM Benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) using a hand-held homogenizer. The homogenate was rotated at 4°C for 1 h, centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatant was collected. Total protein concentration was identified using a BCA protein assay kit (Pierce, Rockford, IL), according to the manufacturer's instructions. This supernatant was then diluted to 1,200  $\mu$ g/ml. The supplied standards were diluted according to the manufacturer's instructions for running the plate with the high sensitivity range (High PMT), which equated to 0.3–4,182 pg/ml for IL-6, 0.1–2,353 pg/ml for IL-8, 0.2–2,718 pg/ml for MCP-1, 0.6–9,176 pg/ml for TNF- $\alpha$ , 0.03 – 503 pg/ml for IL-4, 0.17 – 2,777 pg/ml for IL-10 and 0.15 – 2,828 pg/ml for IL-13. The plate was then read on the Bio-Plex Suspension Array System (V.5.0, Bio-Rad). All samples were run in triplicate, using the High PMT function. Average intra-assay CV% was as follows: 10.6% for IL-6, 5.7% for IL-8, 5.6% for MCP-1, 12.1% for TNF- $\alpha$ , 12.6% for IL-4, 6.8% for IL-10 and 9.8% for IL-13.

## Statistical analysis

Statistical analysis was performed using GenStat 16<sup>th</sup> Edition (VSN International, Hertfordshire). The effects of age (young vs elderly), training (pre vs post) and exercise (pre vs 2 h post) were tested for significance with an analysis of variance (ANOVA), with data

175 blocked for participant (n=16) and biopsy number (n=4). Cytokine expression data was not  
176 normally distributed and, therefore, was analyzed following a  $\log_{10}(x+1)$  transformation.  
177 Data is presented as mean  $\pm$  SEM prior to transformation. A probability level of  $p<0.05$  was  
178 adopted throughout to determine statistical significance, unless otherwise stated.

## Results

### *Muscle Strength and Performance*

Muscle strength variables measured before and after training are presented in Table 2. Isokinetic strength ( $p=0.02$ ,  $p=0.048$ , for concentric and eccentric, respectively) and 1-RM values were all lower in the elderly men compared with the young men. Following 12 weeks of resistance exercise training, all measured strength variables were significantly higher than pre-training values in both groups. The gains in strength as a percentage of pre-training values were comparable between the young and elderly men.

### *Skeletal muscle myokine expression at rest*

Inflammatory and anti-inflammatory cytokine expression in resting skeletal muscle was not significantly different between groups before or following training (Figures 1 & 2). However, IL-6 tended to be greater in the elderly cohort prior to training ( $p=0.07$ ), while this difference was smaller following training (Figure 1A). Considerable inter-subject variation in all pro-inflammatory cytokines was evident, particularly in the elderly cohort.

### *Skeletal muscle myokine expression following maximal isokinetic exercise*

Figure 3 demonstrates the marked activation of pro-inflammatory cytokines at 2 h following each bout of isokinetic exercise before and after 12 weeks of training. The protein abundance of IL-6, IL-8 and MCP-1 increased following each exercise session (Figure 3A-C, significant main effect for exercise  $p<0.01$  for all factors). Although not significant, a trend towards an age  $\times$  training  $\times$  exercise interaction was observed for the expression of MCP-1 ( $p=0.063$ ) and IL-8 ( $p=0.089$ ). The increase in these factors after the pre-training exercise bout was much greater in the elderly men compared with the young men, with larger

increases observed for IL-8 (63 fold in elderly vs. 28 fold in young, Figure 3B) and MCP-1 (26 fold in elderly vs. 9 fold in young, Figure 3C). However, these differences were not statistically significant. Following 12 weeks of resistance exercise training, the exercise-induced increases in MCP-1, IL-8 and IL-6 were similar between the groups.

In contrast to the pro-inflammatory cytokines, the expression of anti-inflammatory cytokines, including IL-10, IL-4, IL-13 (Figure 4A-C) showed more modest changes in response to isokinetic exercise. A significant main effect for exercise was observed for IL-10 ( $p=0.023$ ; 1.12-fold irrespective of age or training status, Figure 4B). IL-4 displayed a significant training  $\times$  exercise interaction, indicating that training elicited exercise-induced expression of IL-4. Prior to training, expression of IL-4 decreased slightly following isokinetic exercise (1.4 fold decrease irrespective of age, Figure 4A). Following exercise training, IL-4 expression was significantly higher 2 h after isokinetic exercise (1.7-fold irrespective of age, Figure 4A). No significant changes were observed for IL-13.

TNF- $\alpha$  was undetectable in 21 out of the 28 samples, and could not be quantified.

## Discussion

The primary aim of the current study was to compare changes in the protein expression of cytokines classically defined as either pro-inflammatory or anti-inflammatory within skeletal muscle of young and elderly men following a single bout of isokinetic exercise. A secondary aim was to investigate whether 12 weeks of resistance training modified these cytokine responses to acute exercise. Before the training period, exercise-induced changes in IL-6, IL-8 and MCP-1 expression in skeletal muscle tended to be greater in the elderly men. Training tended to attenuate these exercise-induced changes, albeit not significantly. These results suggest that ageing is associated with a heightened inflammatory response following unaccustomed exercise and that exercise training may play a role in normalizing this phenomenon.

At rest, the expression of pro- and anti-inflammatory cytokines was generally similar in the young and elderly men—both before and after 12 weeks of resistance training. At rest prior to training, IL-6 expression was 4-fold higher ( $p=0.07$ ) in the elderly men compared with the young men. This difference was smaller following training (Figure 1A). Here, we have classified IL-6 as a pro-inflammatory cytokine, yet it is recognised that under some conditions, IL-6 also exhibits anti-inflammatory properties (Munoz-Canoves et al., 2013). Although somewhat unexpected, the lack of any significant differences in cytokine at rest before and after training is not unique. Some studies have observed greater cytokine expression within skeletal muscle of older individuals (Buford et al., 2010; Leger et al., 2008; Przybyla et al., 2006; Thalacker-Mercer et al., 2010). In contrast, and in line with the current results, other studies have failed to report significant differences in cytokine expression in skeletal muscle between young and elderly individuals at rest (Hamada et al., 2005; Przybyla

et al., 2006; Trenerry et al., 2008) and TNF- $\alpha$  (Hamada et al., 2005; Raue et al., 2007). The protein expression of cytokines in resting skeletal muscle may reflect the health status, as opposed to the chronological age, of the individual.

The intense isokinetic exercise increased the protein expression of the major chemo-attractant and pro-inflammatory cytokines, including MCP-1 (~16-fold), IL-8 (~68-fold and IL-6 (~4-fold) in both young and elderly subjects. These data are consistent with previous literature reporting marked activation in skeletal muscle of the genes encoding MCP-1 (Chen et al., 2003; Hubal et al., 2008), IL-8 (Louis et al., 2007; Nieman et al., 2004) and IL-6 (Buford et al., 2009; Louis et al., 2007; Nieman et al., 2004) up to 4 h following exercise. Interestingly, there was a trend for a greater increase in the pro-inflammatory cytokine response in the elderly in the untrained state. Training tended to diminish this difference. However, these trends were not statistically significant, given both the variability of the response—particularly in the elderly cohort, and the relatively low subject numbers. These findings are in contrast to previous literature, which has demonstrated a suppression of acute pro-inflammatory responses to acute resistance exercise in elderly individuals. Compared with young individuals, elderly individuals display lower circulating neutrophil counts (Cannon et al., 1990; Cannon et al., 1994), reduced intra-muscular macrophage infiltration (Hamada et al., 2005; Przybyla et al., 2006) and lower gene expression of cytokines in skeletal muscle (Hamada et al., 2005; Przybyla et al., 2006) following exercise. Conversely, in more extreme models of injury or infection (e.g., burn injury, endotoxin exposure, cecal ligation and puncture), the pro-inflammatory immune response is exacerbated with age (Kovacs et al., 2004a; Kovacs et al., 2004b; Merritt et al., 2013; Saito et al., 2003). A greater and more sustained pro-inflammatory response has also been

observed in rats following muscle injury, accompanied by a delay in muscle regeneration (van der Poel et al., 2011). Thus, in addition to expanding the analysis of muscle cytokines into a larger elderly cohort, further studies may be required to investigate the effects of aging on pro-inflammatory responses to mild (e.g., exercise) versus more severe (e.g., trauma and ill-health) muscle damage.

In addition to the pro-inflammatory cytokines, the expression of anti-inflammatory cytokines also changed after isokinetic exercise, although to a much smaller extent. We demonstrate, for the first time, that acute exercise significantly increased the protein expression of IL-10 in skeletal muscle, albeit rather modestly when compared with the changes observed in the pro-inflammatory factors. Because the IL-10 response to exercise was similar in young and elderly men, it is doubtful that the heightened pro-inflammatory response in the elderly men was due to dysregulation of the anti-inflammatory response. Ablation of IL-10 prolongs the Th1/pro-inflammatory response, suppresses the Th2 response, and subsequently delays muscle regeneration (Deng et al., 2012). Thus, IL-10 expression in skeletal muscle following exercise may help to regulate the pro-inflammatory response and subsequent muscle regeneration.

Another novel finding of this study was the training-induced regulation of IL-4 following exercise. Prior to training, acute isokinetic exercise did not significantly alter IL-4 expression. However, following training there was a small, but significant increase in IL-4 expression post-exercise. Although training did not increase the resting expression of IL-4, as has been previously described (Gordon et al., 2012; Prokopchuk et al., 2007), the increased expression of IL-4 in response to exercise following the training period may reflect an

adaptation to support myogenesis. IL-4 directly stimulates myogenesis, because it enhances myoblast fusion in vitro (Horsley et al., 2003). Furthermore, knockout of IL-4 or its receptor (IL-4 $\alpha$ ) in mice results in impaired muscle regeneration, mainly through the impairment of fibro/adipocyte progenitor cell function (Heredia et al., 2013). Taken together, this evidence suggests IL-4 plays an important role in the regulation of muscle stem cell functions in supporting myogenesis. Further studies are required to establish the significance of exercise-induced changes in IL-4 expression in skeletal muscle following training.

In conclusion, intense isokinetic exercise markedly increased the abundance of predominantly pro-inflammatory cytokines, while changes in expression of anti-inflammatory cytokines were more modest. Contrary to our hypothesis, neither age nor training modified the relative expression of pro-inflammatory and anti-inflammatory cytokines within skeletal muscle. The pro-inflammatory responses to acute exercise before training tended to be greater in the elderly men, and this difference tended to diminish with training. However, the high inter-individual variability and the small sample size in the present study limit the ability to draw meaningful conclusions.



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454

455 **Table legends**

456

457 **Table 1.** Subject characteristics (Mean±SE). <sup>a</sup> Significantly different from young male  
458 subjects.

459

460 **Table 2.** Muscle strength before and after 12 weeks of resistance exercise training  
461 (Mean±SE). <sup>a</sup> significant main effect for training ( $p < 0.05$ ); <sup>b</sup> significant main effect for age ( $p$   
462  $< 0.05$ ); \* significantly different from Pre Training ( $p < 0.05$ ); #significantly different from  
463 young group ( $p < 0.05$ ).

464

## Figure Legends

**Figure 1.** Resting protein expression of pro-inflammatory cytokines in young and elderly men. Expression of IL-6 (A), IL-8 (B) and MCP-1 (C) protein in skeletal muscle of young and elderly subjects at rest, before (pre training) and after (post training) 12 weeks of resistance exercise training (Mean  $\pm$  SEM).

**Figure 2.** Resting protein expression of anti-inflammatory cytokines in young and elderly men. Expression of IL-4 (A), IL-10 (B) and IL-13 (C) protein in skeletal muscle of young and elderly subjects at rest, before (pre training) and after (post training) 12 weeks of resistance exercise training (Mean  $\pm$  SEM).

**Figure 3.** Protein expression of pro-inflammatory cytokines following 12 weeks of resistance exercise training in young and elderly men. Expression of IL-6 (A), IL-8 (B) and MCP-1 (C) in skeletal muscle of young and old subjects at rest and 2 hours following acute isokinetic exercise, before and after 12 weeks of chronic resistance exercise training (Mean  $\pm$  SEM). # Significant main effect for exercise  $p < 0.05$ .

**Figure 4.** Protein expression of anti-inflammatory cytokines following 12 weeks of resistance exercise training in young and elderly men. Expression of IL-4 (A), IL-10 (B) and IL-13 (C) in skeletal muscle of young and old subjects at rest and 2 hours following acute isokinetic exercise, before and after 12 weeks of chronic resistance exercise training (Mean  $\pm$  SEM). # Significant main effect for exercise  $p < 0.05$ , \* Significant interaction: training  $\times$  exercise  $p < 0.05$ , <sup>a</sup> Significantly different from Rest value, irrespective of age.